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Structural and Functional Analysis of the Six1 Transcriptional Complex for Anti-Breast Cancer Drug Design

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14. ABSTRACT
Cancer and normal development share many properties. During normal development, genes are activated that stimulate proliferation, migration, invasion, vascularization, and that alter cell survival. These gene products are often lost once organ development is complete. In cancer, many developmental genes are re-activated, stimulating the aforementioned processes out of context. The Six1 gene encodes a transcription factor that induces the expression of a large number of genes that are involved in the proliferation, survival, migration, and invasion of cells during embryonic development. In most tissues, Six1 expression is lost once development is complete. However, Six1 is reactivated in many breast cancers, where as many as 90% of metastatic tumors overexpress the gene. Six1 plays a role in both tumor initiation and metastasis of breast cancers, and its inhibition dramatically diminishes both tumor cell proliferation and metastasis in a number of mouse cancer models. Because Six1 is expressed during embryogenesis, lost in most adult tissues, and re-expressed in tumors, we believe it is an ideal drug target whose inactivation will inhibit tumor cell proliferation and metastasis with limited side effects. Our goal in this proposal is to lay the foundation for developing novel, tumor-specific chemotherapeutic agents for breast cancer. This will be accomplished by coupling the expertise of a cancer/molecular biologist with a structural biologist/biochemist. Within the proposal, we will identify multiple avenues for targeting the Six1 transcriptional complex, and use an innovative rational drug design and complementary high throughput screeing (HTS) approach to identify small molecule inhibitors of the Six1 complex. The Six1 transcriptional complex has never before been clinically targeted- but its inhibition would be expected to inhibit both tumor cell proliferation and metastasis, while sparing normal cells. Such a target is badly needed in breast cancer, where many of the currently used therapies have serious side effects. This research is expected to benefit 50% of breast cancer patients with primary breast tumors and 90% with metastatic tumors, as Six1 is expressed in the aforementioned percentage of breast cancer cases. We project to have lead compounds targeting Six1 within five years. The DOD Synergistic IDEA grant will greatly facilitate our efforts in generating lead compounds as soon as possible.

15. SUBJECT TERMS
Breast Cancer Research Program

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INTRODUCTION

In this proposal we are performing structural and functional analyses of the Six1 transcriptional complex for anti-breast cancer drug design. Six1 is a transcription factor that has never before been clinically targeted and that plays a critical role in the onset and progression of a significant proportion of breast cancers. Six1 expression is low or undetectable in normal breast tissue but the gene is overexpressed in 50% of primary breast tumors and 90% of metastatic lesions. Furthermore, examination of public microarray databases containing more than 580 breast cancer samples demonstrates that it correlates significantly with shortened time to relapse, shortened time to metastasis, and decreased overall survival. Using mouse models of mammary cancer, we have demonstrated that its overexpression results in enhanced proliferation, transformation, increased tumor volume, *and* metastasis. Importantly, RNA interference against Six1 decreases cancer cell proliferation and metastases in several different cancer models. The Eya proteins are co-activators of Six1 that utilize their intrinsic phosphatase activity to switch the Six1 transcriptional complex from a repressor to an activator complex. The Six1-Eya interaction is essential for proliferation during embryonic development, and both Six1 and Eya2 have been independently implicated in the same types of cancer. Furthermore, coordinate overexpression of Six1and Eya2 significantly correlates with a dramatically shortened time to relapse and with shortened survival in breast cancers. These findings suggest that Eya and Six1 cooperate to stimulate breast tumorigenesis *and* progression. Because the Eya co-activator contains a unique protein phosphatase domain whose activity is required to activate Six1, it may serve as a novel anti-cancer drug target. However, an essential role for the Six1/Eya interaction and Eya's phosphatase activity in cancer cell proliferation and/or metastasis has not been formally proven. The above observations lead us to hypothesize that the Six1/Eya/DNA complex is an ideal drug target whose inactivation will inhibit tumor cell proliferation and metastasis in breast cancer. Because Six1 and Eya are embryonic genes with very limited expression in the adult, inhibitors of their expression/activity are likely to have limited side effects. To test this hypothesis, this proposal combines my (Dr. Ford's) strength in breast cancer biology with Dr. Rui Zhao's strength in structural biology/biochemistry. To determine whether Six1 activity can be targeted by modulating proteins within its transcriptional complex, we have begun to perform *in vitro* and *in vivo* assays to identify whether the co-factor of Six1, Eya2, and its phosphatase activity, is absolutely required for the ability of Six1 to induce tumorigenesis and metastasis. We are also attempting to solve the X-ray structure of the Six1/Eya/DNA ternary complex with the goal of setting the groundwork for designing structure-based inhibitors. Finally, to ensure that we will obtain inhibitors of the complex, we will perform high throughput screens (HTS) as a second method to identify small molecules that target the Six1-DNA or the Six1-Eya interaction, as well as Eya's phosphatase activity.

BODY

In the body of this progress report, we outline the progress made to date on each task of the original grant.

Task 1. Determine the role of Eya2 and its phosphatase activity in Six1-mediated breast tumorigenesis and metastasis (years 1 and 2). Work carried out in Dr. Ford's laboratory.

1A. Determine the effect of Eya2 knockdown on Six1-induced proliferation (months 1-6)

In this sub-aim, we wanted to determine whether loss of Eya2 in Six1 overexpressing MCF7 cells led to a decrease in proliferation. Previously, we had shown that proliferation was increased with Six1 overexpression [1] and that this increase was dependent on cyclin A1 activation by Six1. Preliminary experiments with Eya2 knockdown demonstrated that Six1 did depend on Eya2 for its ability to activate cyclin A1 (Fig.1). However, over time our Six1-overexpressing cells have changed such that they no longer proliferate more rapidly than the control cells in culture. We believe that this change may be due to the epithelial to mesenchymal transition (EMT) that the cells underwent in the presence of Six1-overexpression that occurred over time. Thus, we were unable to assess whether Eya2 knockdown reverses Six1-induced proliferation, as Six1-induced proliferation was lost in these cell lines. However, we were able to assess the role of Eya2 in Six1-induced EMT, a property that is associated with metastasis and will be outlined in Aim 1B below.

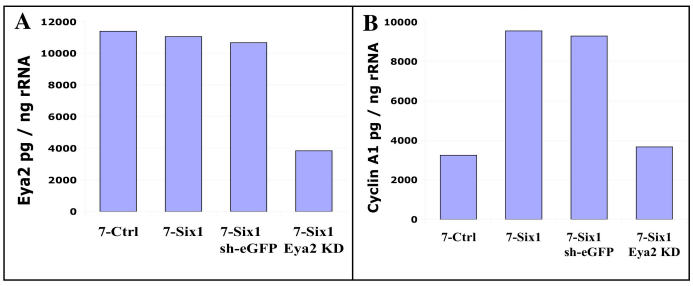

Fig. 1. Eya2 shRNA efficiently knocks down Eya2 in MCF7 Six1 cells, leading to a decrease in cyclin A1 levels of mRNAs determined by qRT-PCR.

1B. Determine the effect of Eya2 knockdown on transformation, tumor burden and metastasis (months 1-18)

We have made significant progress on this sub-aim, and are just in the process of writing our first manuscript that demonstrates that Eya2 is required for the ability of Six1 to mediate increased TGF-β signaling, EMT, increased stem cell capacity, and increased tumor initiating capacity. In this sub-aim, we stably knocked down Eya2 is MCF7-Six1 expressing cells and compared the Eya2 knockdown cells to MCF7-Six1 cells with a control shRNA and to MCF7-control (Ctrl) cells with a control shRNA. Fig. 2 shows the relative levels of Eya2 mRNA expression after real time RT-PCR in the cell lines containing shRNAs (2 different shRNAs were used, 2 clonal isolates were generally analyzed for each shRNA) vs control cell lines. As is evidenced from Fig. 2, the shRNAs targeting Eya2 effectively knocked the gene down in Six1-overexpressing cells.

We have recently demonstrated that Six1 mediates metastasis via its ability to upregulate TGF-β signaling [2]. In addition, we have also shown that Six1 increases TGF-β signaling at least in part via upregulating the type 1 TGF-β receptor, TβRI (Micalizzi et al., under review at Cancer Res 2010). We thus asked whether knockdown of Eya2 in Six1-overexpressing MCF7 cells could reverse the ability of Six1 to increase TβRI levels, and to activate TGF-β signaling. Indeed, Figure 3 demonstrates that Eya2 knockdown in Six1 overexpressing cells reverses the ability of

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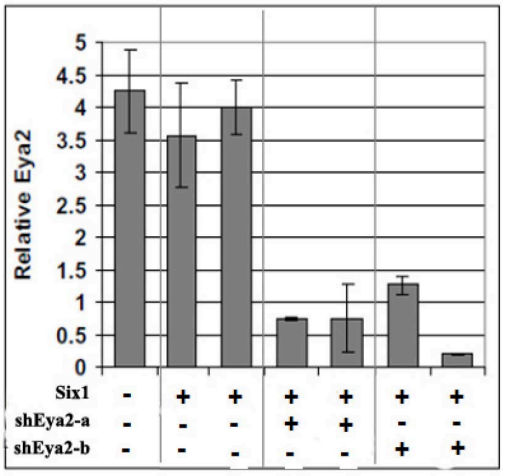

Figure 2: Eya2 expression is efficiently knocked-down in MCF7-Six1. Real time RT-PCR of stable Eya2 and scramble shRNA clones. Two clonal isolates were chosen for analysis from each shRNA group with two separate shRNA constructs targeting Eya2 (only one was chosen in the control, non-Six1 expressing cells. Error bars represent the standard deviation of the mean of triplicate RNA

Six1

increase TβRI levels, to increase both total Smad3 and phospho-Smad3 levels, and to activate Smad-mediated transcription, as assessed by the 3TP Smad-dependent reporter luciferase assay. Together, these data clearly demonstrate that Six1 requires Eya2 to activate TGF-β signaling.

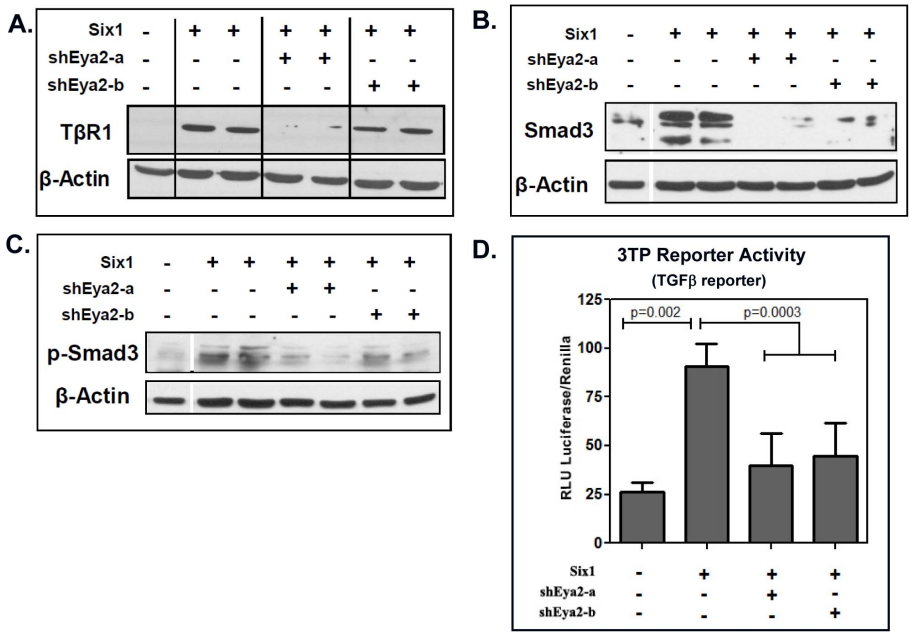

Figure 3: Loss of Eya2 in MCF7-Six1 cells reverses Six1-induced TGFβ signaling. Six1 overexpressing cells containing an Eya2 shRNA have decreased levels of (a) TβR1, (b) total Smad3, and (c) phosphorylated Smad3 protein. (d) Eya2 shRNA cells showed decreased TGFβ-responsive transcription compared to Six1 scrambled controls. Responsiveness was tested using luciferase activity of the 3TP reporter construct and normalized to renilla activity. Data points show the mean of two individual clones and error bars represent the standard error of the mean for 2 experiments. *P* values represent unpaired *t* test statistical analysis.

Because we had previously demonstrated that Six1 induces an EMT in MCF7 cells that is dependent on its ability to activate TGF-β signaling, we went on to examine the role of Eya2 in Six1-induced EMT. Importantly, knockdown of Eya2 in Six1 expressing cells reverses the ability of Six1 to lead to an increase in the mesenchymal protein fibronectin, and reverses the ability of Six1 to re-localize E-cadherin and β-catenin, two adherens junction proteins, away from the membrane and into the cytosol (Fig 4A and B). Furthermore, Six1 is also dependent on Eya2 for its ability to lead to an increase in β-catenin mediated transcription, a hallmark of EMT (Fig. 4C). Interestingly, however, Eya2 knockdown did not reverse the ability of Six1 to lead to decreased cell-matrix adhesion (Fig. 4C), a property that was also not reversed in Six1

overexpressing cells in which TGF-β signaling was downregulated (Micalizzi et al., under review at Cancer Res 2010). These data strongly support a role for Eya2 in some, but not all properties of Six1-induced EMT, and suggest that Eya2 cooperates with Six1 to induce EMT phenotypes that are dependent on TGF-β signaling.

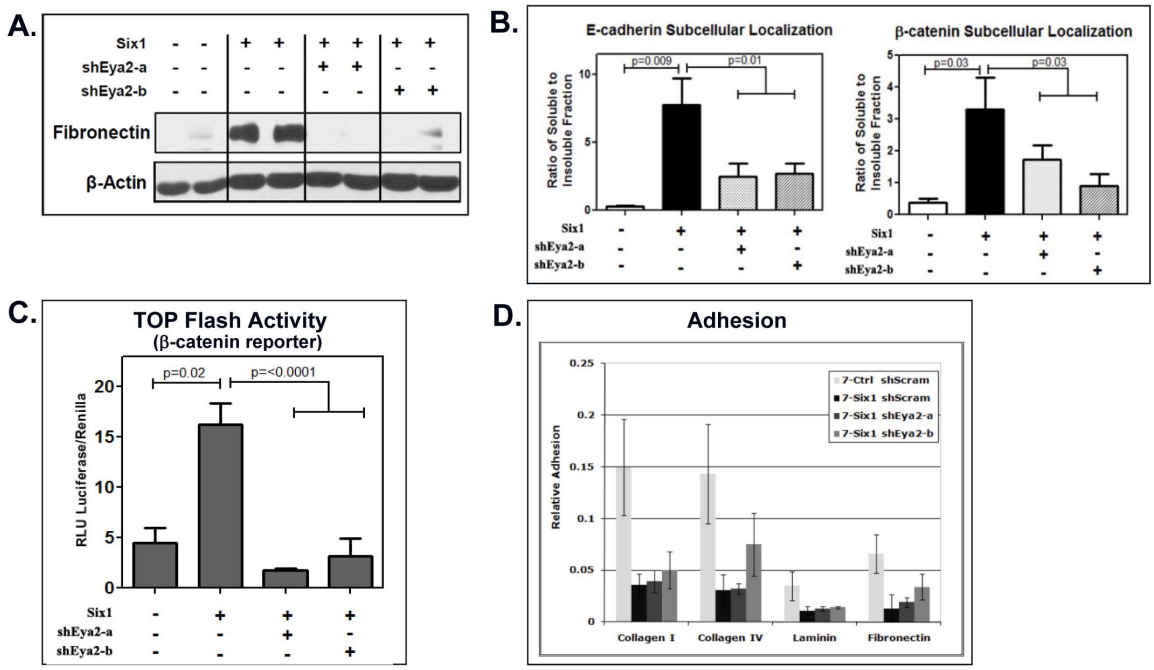

Figure 4: Loss of Eya2 in MCF7-Six1 cells reverses most Six1-induced properties of EMT. Six1 overexpressing cells containing an Eya2 shRNA (a) reverse the ability of Six1 to upregulate the mesenchymal marker Fibronectin and (b) reverse the ability of Six1 to relocalize E-cadherin and β-catenin from the insoluble (membranous) fraction to the soluble (cytoplasmic) fraction as shown by western blot quantification following cell fractionation. Western blot analysis was performed on whole cell lysates using Fibronectin, E-cadherin, β-catenin, and β-actin antibodies. (c) Eya2 shRNA clones have decreased β-catenin responsive transcription compared to Six1 scrambled controls. Responsiveness was tested using luciferase activity of the TOP-flash reporter construct and normalized to renilla activity. Data points for fractionation and reporter activity show the mean of two individual clones and error bars represent the standard error of the mean for 2 experiments. *P* values represent unpaired *t* test statistical analysis. (d) Eya2 shRNA clones show the same decreased cell-matrix adhesion as Six1 overexpressing cells. Relative adherence was measured by crystal violet staining.

Because genes that induce EMT often also induce stem cell characteristics, and because we have obtained data that demonstrates that Six1 overexpression in MCF7 cells leads to increased cancer stem cells, as measured by flow cytometry, mammosphere assays, and *in vivo* tumor initiating transplant assays (Ritsuko and Ford, manuscript in preparation), we asked whether Six1 was dependent on Eya2 to induce cancer stem cell characteristics. Indeed, knockdown of Eya2 in Six1-overexpressing cells reversed the increase in the breast cancer stem/progenitor pool as measured by flow cytometry for the $CD44^{+}/CD24^{lo}$ stem cell population (Fig. 5A). It also reversed the ability of Six1 to lead to increased mammosphere formation, an *in*

vitro test for functional stem/progenitor cells (Fig. 5B). Currently, we are examining whether Six1 is dependent on Eya2 to increase the cancer stem cell pool using in vivo, serial dilution transplant assays, which is the final test needed to conclusively demonstrate that Eya2 does influence the ability of Six1 to lead to increased cancer stem cells.

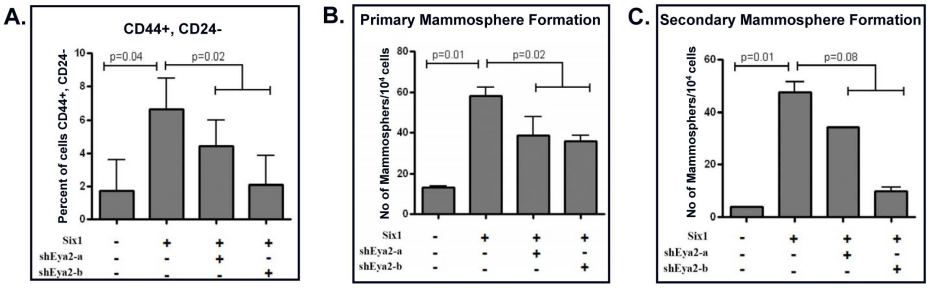

Figure 5:. Six1 overexpressing cells containing an Eya2 shRNA have decreased stem/progenitor cell characteristics. (a) Flow cytometric analysis shows loss of stem cells with loss of Eya2. (b & c) Primary and secondary mammosphere assays show decreased mammosphere formation with loss of Eya2. Antibodies used to perform flow cytometry include CD24 and CD44, markers found on human epithelial stem cells. Data points show the mean of two individual clones and error bars represent the standard error of the mean for 2 experiments. *P* values represent unpaired *t* test statistical analysis.

We have also examined the dependence of Six1 on Eya2 in human breast cancers. By examining the Van de Vijver dataset of 295 breast cancer patients with early-stage invasive carcinoma [3], we have observed that while high Six1 in the absence of high Eya2 or high Eya2 in the absence of high Six1 do not predict shortened time to relapse, metastasis, or survival, having both high levels of Six1 AND Eya2 together do significantly correlate with shortened time to relapse and metastasis, and with shortened overall survival (Fig. 6). Indeed, these data are recapitulated in the Wang dataset of 286 node-negative breast cancers [4](data not shown). Together, these data strongly suggest

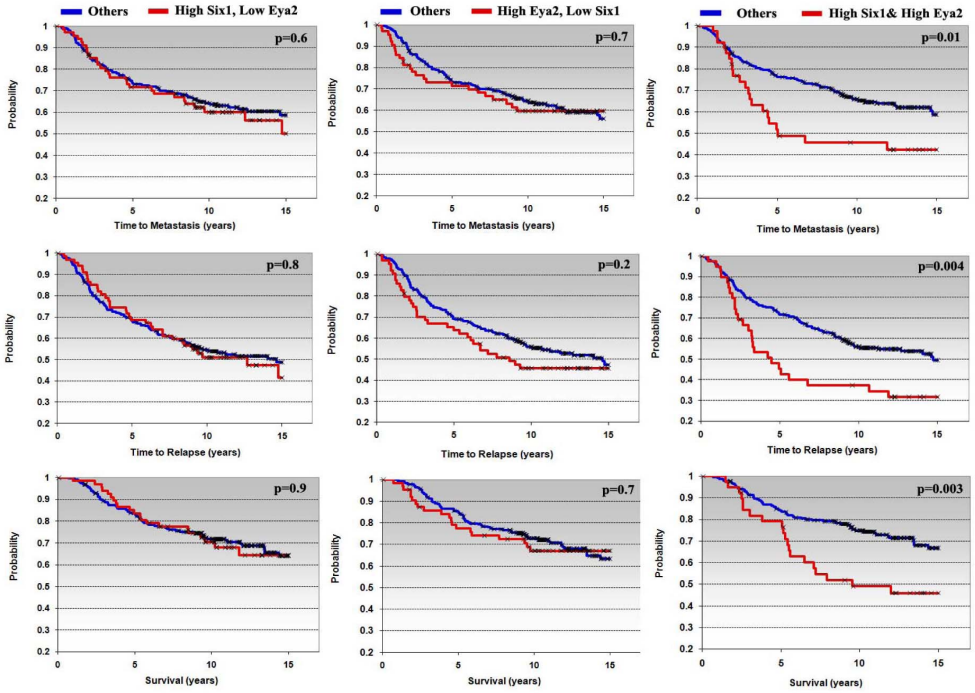

Figure 6: Presence of both high Six1 and high Eya2 correlates with adverse outcomes. In a microarray analysis of 295 women with early-stage invasive breast carcinoma[3], expression of both high Six1 & high Eya2 in the same tumor sample correlates with (a) reduced time to metastasis, (b) reduced time to relapse, and (c) shortened breast cancer-specific survival. The median value for Six1 and/or Eya2 expression was used to divide the samples into high (above the median) and low (below the median) Six1 and and Eya2 expressers. P-values were calculated by log-rank analysis.

that in human breast cancers, like in our model systems, Six1 is dependent on Eya2 to mediate its pro-tumorigenic and metastatic phenotypes.

1C. Determine whether Eya's phosphatase activity is required for Eya's effect on Six1-mediated breast tumorigenesis/metastasis (months 12-24).

We are a little ahead of schedule in that we have already made the phosphatase dead mutant of Eya2. We are currently transducing Six1-overexpressing Eya2 knockdown cells with a wild type Eya2, and with an Eya2 phosphatase dead mutant (active site mutant Eya2 D278N, which interferes with the Eya2 tyrosine phosphatase activity). Once our cell lines have been generated, we will begin to ask whether the phosphatase activity of Eya2 is required for Six1-mediated tumorigenic and metastatic phenotypes.

Task 2. Identify small molecules that inhibit the Six1/Eya/DNA complex using structure-based and high throughput screen (HTS) approaches

This aim is primarily carried out by Dr. Zhao's laboratory with the one exception that any identified small molecules will be tested in cell culture by Dr. Ford's laboratory (they will first be tested biochemically by Dr. Zhao)

2A. Determine the crystal structure of Six1/Eya/DNA ternary complex for structure-based drug design

We have generated 22 Six1 constructs (4 truncation and 18 mutations) and two different Eya constructs. We have expressed and purified 6 of the Six1 constructs, the two Eya constructs, and will continue to express and purify the remaining Six1 and Eya constructs. We are in the process of setting up crystallization trials with these constructs in an attempt to improve crystals.

In addition, we have obtained crystals of the Six1/Eya complex (Fig. 7). We are in the process of improving these crystals. These crystals provide an important alternative in case we have difficulty improving the Six1/Eya/DNA crystals. The Six1/Eya structure will elucidate the molecular details of the Six1/Eya interaction, which will be valuable for targeting the Six1/Eya interaction using structure-based drug design approach.

Fig. 7. Crystals of the Six1/ED complex.

2B. **Identify small molecules that inhibit the Six1/Eya/DNA complex using HTS**

We have mostly focused on developing an Alpha-Screen assay for targeting the Six1/Eya protein/protein interaction in the past year. Our preliminary results show that Six1 and Eya together generate a significant fluorescence signal in the Alpha-Screen assay while Six1 or Eya alone does not (Fig. 8). We are in the process of performing various controls to ensure the specificity of the assay and adapting it to a high throughput screening format.

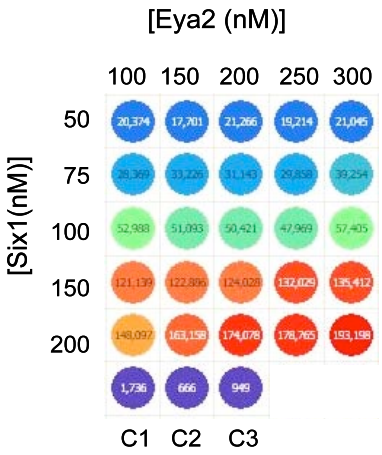

Fig. 8. Fluorescence reading with increasing concentration of Six1 (top to bottom) and Eya (left to right) in an AlphaScreen assay. Wells C1-C3 are controls that contains 200nM Six1 alone (C1), AlphaScreen donor and receptor beads alone (C2), and buffer alone (C3).

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that Eya2 is required for Six1-induced TGF-b signaling
- Demonstration that Eya2 is required for many of the Six1-induced EMT properties
- Demonstration that Eya2 is required for Six1-induced cancer stem cell characteristics
- Demonstration that Eya2 and Six1 together correlate with adverse outcomes in breast cancer
- Demonstration that Six1 and Eya2 ED can form crystals
- Demonstration that AlphaScreen is a promising assay for high throughput screening targeting Six1 and Eya's protein-protein interaction

REPORTABLE OUTCOMES

Presentations (Susan Farabaugh, graduate student on project):

1. AACR Frontiers in Basic Research, October 2009
2. Annual Student Research Symposium, University of Colorado (one of the top prize winners for this presentation).

Degrees obtained

1. Aaron Patrick obtained PhD in November 2009 from the Molecular Biology Program at The University of Colorado School of Medicine in part on his crystallography work performed for this proposal.

Currently, we are preparing one manuscript on the importance of Eya2 for Six1-mediated phenotypes.

CONCLUSION

To date, our data clearly demonstrate that Eya2 is required for the ability of Six1 to induce both tumorigenic and metastatic properties. These data are important because Eya2 may make a very good anti-breast cancer drug target. Because Six1 is a transcription factor, it will likely not be targetable with small molecule inhibitors. However, since Eya2 is required for Six1-mediated effects, we may be able to target the Six1/Eya interface as an anti-breast cancer therapy. More importantly, work outlined for the coming year will determine whether the Eya2 phosphatase activity is required for Six1-mediated tumorigenesis/metastasis. If it is, the phosphatase activity will be an even better drug target for small molecule inhibitors. In fact, as outlined in the progress report for Aim2, we have already begun to identify some Eya2 phosphatase inhibitors, suggesting we are on our way to identifying new small molecules to target breast cancer.

Targeting the Six1/Eya complex is novel because this complex is critical for normal embryonic development, but is not believed to be required by adult, differentiated cells (in fact, the two proteins are not expressed in many adult tissues). Thus, targeting this transcriptional complex has the ability to inhibit the tumor on multiple fronts while conferring limited side effects. Such breast cancer targets are badly needed and it is for this reason that we are interested in carrying out the work described in this proposal.

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1 Coletta, R. D. *et al.* The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. *Proc Natl Acad Sci U S A* **101**, 6478-6483 (2004).

2 Micalizzi, D. S. *et al.* The Six1 homeoprotein induces human mammary carcinoma cells to undergo epithelial-mesenchymal transition and metastasis in mice through increasing TGF-beta signaling. *J Clin Invest* **119**, 2678-2690, doi:37815 [pii] 10.1172/JCI37815 (2009).

3 van de Vijver, M. J. *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* **347**, 1999-2009 (2002).

4 Wang, Y. *et al.* Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* **365**, 671-679 (2005).

Principal Investigator Assurance

♦ I assure that I have involved the Facility Safety Director/Manager in the planning of this research proposal, discussed with him/her all aspects of the proposal that relate to occupational health and safety, and will help him/her prepare the annual Facility Safety Plan Status Report.

♦ I assure that I will comply with my institution's safety program and its requirements.

♦ I understand that I am directly responsible for all aspects of safety and occupational health specific to my research protocol.

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♦ I assure that hazards associated with my research have been identified eliminated and/or controlled.

♦ I assure that all Facility Safety Plan requirements are in compliance with Local, State and Federal general industry standards.

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